TYROSIN IN THE FUNGI: CHEMISTRY AND METHODS OF STUDYING THE TYROSINASE REACTION

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INTRODUCTION

That certain fungi turn blue or black on exposure to the air and that this property is destroyed by heat has been known for many years, Pallas (1771), Bonnet (1781), Saladin (1779), and Bulliard (1791) having incidentally recorded observations of the fact. Macaire (1824), in a very extended memoir on the subject, brought out nearly all the main lines of proof of the essential features of the reactions involved, although some of his data are better explained on the theory of enzyme action which has developed since his time. Since his work many additional facts have been ascertained, as may be seen by the excellent review papers by Kastle ('10), Clark ('10, '11), and Bach ('13), as well as by others of the Geneva workers to be mentioned later. All of the work so far has dealt almost exclusively with the formation of pigment, and practically nothing is known of the actual chemical changes beyond the fact proved by Macaire that oxygen was absorbed and suggestions as to a partial oxidation of the side chain in the case of tyrosin.

This being the general situation, an attempt has been made to determine some of the chemical changes taking place in the reaction. After an extensive study of the available literature, it seemed best to attack the problem of the tyrosinase reaction first, since this enzyme is more specific and much more is known regarding the products of oxidation as they have been studied in the animal organism.

The outstanding fact in the chemistry of tyrosin is its low solubility (1:2500), which necessitates refining many of the ordinary chemical procedures in order to work with the acid itself, not its sodium salt nor its hydrochloride. Work with the acid would enable one to work with the substrate much nearer the neutral point than would have been possible otherwise, thus avoiding interfering conditions due to hydrogen ion concentrations other than that of water.

The literature on the oxidases in general, including most of the papers on the tyrosinase reaction, has been so excellently reviewed by Behrens ('07), Kastle ('10), Clark ('10, '11), Bach ('13), Rose ('17), and probably Schweizer ('16), that there is little need to add to the reviews already published, although certain works will be discussed in some detail in connection with the methods employed and the results obtained.

I wish at this point to acknowledge indebtedness to those who have so kindly offered suggestions as the problem has developed. Thus, I wish to thank the Missouri Botanical Garden and the Medical School of Washington University for the use of library and laboratory facilities; Dr. B. M. Duggar, under whose supervision the work has been done and without whose advice it would have been difficult indeed; Dr. P. A. Shaffer, Dr. Lucien J. Morris, and Dr. E. R. Allen, all of the Medical School, for valuable suggestions regarding chemical procedures.

Methods

Extraction and precipitation of the enzyme.—Of the many methods used for the extraction of the enzyme and its separation from laccase, fractional precipitation has been most widely used (Chodat, '10, Wohlgemuth, '13). The methods previously reported by Bertrand ('96), Bach ('08, '10), Chodat and Staub ('07), Bertrand and Muttermilch ('07), von Fürth and Schneider ('01), and von Fürth and Jerusalem ('07), were tried out, but for the conditions under which I worked none proved especially satisfactory. I was unable to filter rapidly enough to prevent the inactivation of the enzyme by its long contact with the precipitant. The use of the sap before precipitation was tried, but this was not very satisfactory, since it necessitated diluting the tyrosin and thus adding to the already great difficulties of analysis, owing to the extreme insolubility of the product. Therefore in the work with tyrosin, the dried fungous flour was added directly to the substrate, toluol added, and the mixture left to extract the enzyme and the enzyme to react with the tyrosin, a general method used by Zeller ('17). In work with phenylalanin and other amino-acids which have a much greater solubility than tyrosin, the calculated amount of fungous flour was extracted with chloroform water for 24 hours, filtered, and the filtrate mixed with an equal volume of N/250 of the amino-acid used, thus making the final concentration N/500, approximately that of a saturated solution of tyrosin, and allowing the same chemical procedures to be followed.

The fungi were brought in from the collecting trip, cut up by a vegetable slicer into pieces about 1–2 mm. thick, spread out on the table top, and allowed to dry at room temperature, either with or without an electric fan to keep the air in circulation. Some, such as *Daedalea confragosa,* which are coriaceous, were treated alternately with two or three volumes of 95 per cent alcohol and acetone until most of the water was removed from the tissues, then dehydrated with absolute alcohol and dried as above, in order to facilitate grinding by rendering the tissue more brittle. The fungous "chips," resembling the potato chips of commerce, were ground in a large mill to about the fineness of wheat bran. This was sifted through an 80-mesh sieve and the powder stored in glass bottles, which were sealed with paraffin until needed. The bran was likewise stored in bottles awaiting a chemical study. The fungi were obtained in the fall of 1916, except the material of *Polyporus sulphureus,* of which about 10 kilos fresh weight were obtained in the fall of 1917.

Methods previously employed in the study of the tyrosinase reaction.—Most of the work done on the tyrosinase reaction has been qualitative, where the enzyme has been allowed to come into contact with the substrate, and the resulting colors noted.

Five kinds of quantitative methods have been used in the study of this reaction. Von Fürth and Jerusalem ('07) have developed a spectrophotometric method, and also a sedimentation method by which the volume of the precipitate is measured in graduated centrifuge tubes, but neither seemed to give wholly consistent and reliable results and the methods were not tried out.

A third method was proposed by Bach ('08[a]), in which the colored solution was titrated by N/500 potassium permanganate until the color disappeared. This method was tried out early in this work, but I was unable to titrate to a definite end point. Perhaps had I had the experience in matching colors which was developed later in connection with the colorimetric method, I might have secured more uniform results such as Bach was able to obtain. As with many methods, the personal equation plays a large part in the accuracy obtainable.

A fourth method was that used by Chodat and Staub ('07) and by Staub ('08), in which a series of standards were made up by means of Bismarck brown and corallin to fit the usual degrees of color change and were compared with the unknown. This method gave them comparative results, but was hardly suited to my purpose.

The last set of methods was employed in the gas exchange work, usually limited to a study of the amount of gas absorbed. The method of Bunzel ('12, '14) was tried with the commercial form of his apparatus, but was wholly unsuccessful. The apparatus is too small to admit enough of the tyrosin solution to absorb a measurable amount of oxygen. The McNair modification of the Van Slyke apparatus for the determination of amino-nitrogen is much more useful in every way and gives more consistent results. The procedure was modified slightly for rapidity of determination, as will be described later.

Methods used in the present paper.—In this study an attempt was made to determine the amounts of the various reacting portions of the tyrosin molecule, thus hoping to secure a suggestion as to the actual chemical changes taking place in the formation of "melanin" and other pigments, due to the action of tyrosinase on tyrosin. A glance at the structural formula of tyrosin shows that there are three readily reacting groups for which more or less well-defined methods of determination exist. These are the amino group, which may be determined by the Van Slyke method, the carboxyl group, which may be determined by titration, and the phenol group, which may also be titrated by a suitable method. If ammonia is split off, this is easily determined in several ways.

Determination of amino-nitrogen.—The "micro" Van Slyke apparatus, with certain modifications, was used for the determination in the earlier experiments. By taking extreme care in manipulation, 8 cc. of the solution analyzed could be used in a determination instead of the usual 2 cc. In this case it was convenient to use 60 per cent sodium nitrite solution instead of 30 per cent. Caprylic alcohol was employed occasionally if the mixture started to foam. Later it was found possible to use a "micro" burette with the "macro" form of apparatus, providing proper attention was paid to the amount of nitrogen liberated from the nitrite in the check. It was noted that after all of the air was expelled from the apparatus, successive buretteful of the oxides of nitrogen gave a series of readings as follows: 0.12 cc., 0.10 cc., 0.10 cc., 0.10 cc., 0.09 cc., 0.10 cc. Much care must be taken to be sure that the alkaline permanganate solution is absorbing properly. In the usual procedure, all of the nitrogen is contained in the first buretteful, or in the first few cubic centimeters of the second buretteful, hence the error due to not taking into consideration the total gas volume before absorption was negligible. However, when dealing with such a dilute solution, this precaution was found necessary, as the amount of nitrite is in such large excess of the amount actually needed for the reaction.

Determination of ammonia.—The permutit method of ammonia determination as described by Folin and Bell ('17) was found to be adequate for our purpose. Two grams of permutit were placed in the bottoms of each of the required number of 100-cc. volumetric flasks, the solution to be analyzed or the standard, as the case might be, was added, and the flasks shaken by a mechanical shaker for 5 minutes.

The liquid was decanted, the permutit covered with ammonia-free water, and this decanted and the action repeated. Then 5 cc. 10 per cent NaOH were added, the flasks shaken, about 60 cc. of water poured in, and the flasks shaken again to mix thoroughly, thus preventing the alkali being too concentrated for the Nessler reagent. Ten cubic centimeters of the Nessler reagent, prepared by the method of Folin and Denis ('16), were added, the flasks shaken, allowed to stand 15 minutes and made up to the mark, then read in a Kober ('17) colorimeter. An attempt was made to have the standard contain the same amount of ammonia as the solutions analyzed, but the other errors involved in such extremely dilute solutions were so great that exactness in this respect was not attempted. Artificial light was used, also the long cups, giving approximately 80 mm. working depth. A rheostat, by which the light could be dimmed, was found to be an advantage in working with such dilute solutions, as the eye distinguishes more readily between solutions which appear dark-colored than between very light ones. The same applies to the micro-titration method about to be described.

Determination of carboxyl and phenol groups.—The formol titration method as developed by Sörensen ('07) and by Sörensen and Jessen-Hansen ('08) and extensively used since for the determination of the carboxyl of amino-acids was tried with no better success than Sörensen obtained with tyrosin, and after attempting several modifications it was abandoned. Upon the addition of the formalin solution (prepared by bubbling the formaldehyde given off from paraformaldehyde during heating, into double distilled water) the indicator, thymolsulphonphthalein, if present in the usual amount (5 drops), was quickly decolorized, especially in the presence of the first drop of alkali added. Upon the addition of more indicator, the solution finally turned a dirty, purplish red, very different from that obtained with this indicator at any known hydrogen ion concentration, so that comparison was impossible. The pigment present did not seem to have any color changes, at least in the region in which one must work with tyrosin, thus making thymolsulphonphthalein, the only indicator which I have found which changes at the right point for work with tyrosin, valueless. Sörensen ('07) states that he was unable to use either phenolphthalein or thymolphthalein in the formol titration of tyrosin.

A method was then devised for the titration of dark-colored solutions, by titrating them until they matched a solution of definite hydrogen ion concentration, thus making possible a graph after the method of Bovie ('15), which will be described more fully in connection with the discussion of results. The basic principles of the method were worked out in connection with a paper by Dr. B. M. Duggar and the writer (Duggar and Dodge, '19). A Kober colorimeter, with artificial light and rheostat for varying the light intensity, was used as in the ammonia determinations cited above. The general method of Walpole ('10), improved by Homer ('17), of comparing colored solutions, was adapted to our purpose. For details and precautions necessary in such color comparisons, see paper cited above (Duggar and Dodge, '19). It was found after some experiments that for ordinary work the amount of light absorbed by the water was so slight that it might be dispensed with, making the apparatus much easier to manipulate. In this case the right cup was set at 20 mm. and lowered in proportion to the amount of alkali added, as this diluted the color in the cup. This phenomenon was found to make the solutions incomparable as dilution increased, although such a small amount of dilution did not affect the hydrogen ion concentration of the solution appreciably. The burette used consisted of a 2-cc. pipette, graduated to 0.02 cc., melted on a glass stopcock, the end of which was drawn out to a tip which gave drops of 0.01 cc. of N/50 $Ba(OH)_2$ consistently. This tip was placed directly over the liquid so that a drop of water was rarely necessary to wash down the drop. The solution was stirred after the addition of each drop, by agitating the cup about the prism, care being taken to prevent the spilling of the solution.

In the work with tyrosin, thymolsulphonphthalein was found to be the most suitable indicator, since it has an alkali range between P_H 8.0 and 12.0. This same method was extensively employed with other indicators with an acid range in the work with Dr. Duggar (Duggar and Dodge, '19), so that it seems to have a wide application, although considerable practice and an intimate knowledge of the minute color changes of the indicator worked with is required before accurate titrations, or rather series of titrations, can be made rapidly. Five cubic centimeters of solution is a suitable quantity to titrate, and the process of obtaining a graph is essentially as follows: The cups are properly filled with colored solutions, indicator, etc., then the solution is titrated by the addition of a drop at a time until the first hydrogen ion concentration is reached, in the present work P_H 8.2. Then the standard color corresponding to P_H 8.4 is placed in the cup and the unknown titrated until this hydrogen ion concentration is reached. This is recorded and the process repeated until the curve has been carried as far as desirable, or until some disturbing factor enters which makes further titration impossible. Below is a set of titrations taken at random to show the accuracy of the method. Solution A was almost black, solution B was a golden straw color, and solution C was a tyrosin solution, consequently colorless.

Later even a greater accuracy was obtained in the critical region of P_H 9.0 to 9.4, and I do not doubt but that greater practice will enable one to refine the process still further. The difficulty of estimating quantities of less than 0.01 cc., of manipulating the stopcock, and of securing a burette tip which will drop less than 0.01 cc. without being easily broken, makes the probable error of the single observation a better measure of accuracy than percentage.

N/50 $Ba(OH)_2$ was used as the alkali in titrating tyrosin, since it is removed from harmful action other than lowering the amount of free alkali in the solution because of its insolubility, and also the presence of carbonate is more easily detected. As soon as carbonate was noted, the solution was discarded if in the burette, and filtered and restandardized if in the stock bottle, although this precaution was probably unnecessary in view of the experimental error in other direc-

TABLE I

SHOWING THE ACCURACY OF THE COLORIMETRIC TITRATION (ARMILLARIA MELLEA)

Solution	No. cc. N/50 NaOH required for 5 cc. sol. to give P_H values indicated															
P_H values	8.2	8.4	8.6	8.8	9.0	9.2	9.4	9.6	9.8	10.0	10.2	10.4	10.6	10.8	11.2	11.6
.25 gm. fung. meal.	.02	.....	.21	.28	.31	.53	.56	.58	.....	.....	an interfering color developed					
75 cc. tyrosin sol.	.04	.10	.23	.32	.45	.63	.66	.68	.70	.72	.74	.77	.84	1.27	1.37	1.47
(Solution A)	.03	.08	.16	.38	.41	.63	.66	.68	.70	.72	.76	.78	.84	1.32	1.40	.....
.25 gm. fung. meal	.....	.....	.22	.24	.26	.28	.30	.32	.....	.....	.33	.....	.34	.35	.36	.40
75 cc. dist. water	.....	.22	.23	.24	.26	.28	.29	.30	.....	.....	.....	.....	.....	.33	.36	.40
(Solution B)	.....	.22	.24	.25	.26	.28	.29	.30	.....	.....	.....	.....	.....	.31	.32	.34
75 cc. tyrosin sol.	.....	.20	.25	.37	.45	.71	.80	.83	.....	.84	.85	.99	1.07	1.16	1.27	1.35
(.20 gm. in 500 cc.)	.....	.....	.27	.36	.44	.70	.76	.81	.82	.83	.84	.85	.97	1.01	1.06	1.16
(Solution C)	.11	.22	.26	.46	.64	.94	.97	1.03	1.03	1.04	1.07	1.09	1.13	1.51	1.95	2.00
Average titration																
Solution A	.03	.09	.20	.33	.39	.59	.63	.65	.70	.72	.75	.77	.84	1.30	1.38	1.47
Solution B	.....	.22	.23	.24	.26	.28	.29	.31	.31	.31	.31	.31	.31	.33	.35	.38
Solution C†	.....	.21	.26	.36	.44	.70	.78	.82	.82	.83	.84	.91	1.02	1.08	1.16	1.25
Solution C*	.11	.21	.26	.40	.50	.78	.84	.89	.89	.90	.92	.98	1.06	1.23	1.43	1.50
Greatest deviation of the single observation from the average																
Solution A	.01	.01	.04	.05	.08	.06	.07	.07	.00	.00	.01	.00	.00	.02	.01	.....
Solution B	.....	.00	.01	.01	.00	.00	.00	.01	.01	.01	.02	.02	.03	.02	.03	.04
Solution C†	.....	.01	.01	.00	.00	.00	.02	.01	.00	.00	.00	.08	.05	.08	.11	.10
Solution C*	.....	.01	.01	.06	.14	.16	.13	.14	.14	.14	.15	.14	.13	.26	.52	.50

$$\text{Probable error of the single observation} = \pm \frac{\text{greatest deviation of the single observation}}{\sqrt{\text{number of observations}}}$$

	Figures below $\times 10^{-2}$															
Solution A	.58	.71	2.32	2.90	4.64	3.48	4.06	4.06	.00	.00	.71	.29	.00	1.41	.71	.....
Solution B	.....	.00	.58	.58	.00	.00	.17	.58	.58	.58	1.46	1.46	1.74	1.46	1.74	2.32
Solution C†	.....	.71	.71	.35	.35	.35	1.41	.71	.35	.35	.35	5.66	3.50	5.66	7.74	5.80
Solution C*	.....	.58	.58	3.48	8.13	9.29	7.55	8.13	8.13	8.13	8.13	8.71	8.13	7.55	5.10	30.20

† This row of figures shows the results of treating the first two titrations of the tyrosin solution by themselves. The probable error of the single observation is greater owing to the fewer observations.

* This row of figures shows the greatest variation from the other figures and is included merely to show that even with such extreme figures the error is quite small. It will also be noted that while the absolute values vary considerably from those of the other titrations, when plotted they would yield a curve very similar to the other two. This titration was made with a different preparation of tyrosin.

tions. In the work with phenylalanin, on the other hand, N/50 NaOH was used, since, as Sörensen ('07) noted, the comparatively insoluble barium salt formed shows a tendency to adsorb the indicator.

In this work, the solutions of definite hydrogen ion concentration with P_H values between 8.0 and 9.6 were the borate solutions described by Clark and Lubs ('17); beyond P_H 9.6 they were the mixtures described by Sörensen ('09) or those which vary by P_H 0.2, and were made up from glycin, sodium chloride, and sodium hydroxide solutions as accurately as possible from the data given in the large chart which accompanies the French edition of the work. The quantities used are given here, as this edition is less readily available.

Solution	No. cc. necessary to give P_H values indicated												
P_H values......	9.6	9.8	10.0	10.2	10.4	10.6	10.8	11.0	11.2	11.4	11.6	11.8	12.0
NaOH.........	27.0	32.0	37.0	41.0	44.0	46.0	47.5	48.8	49.5	50.4	51.2	52.2	54.0
Glycin.........	73.0	68.0	63.0	59.0	56.0	54.0	52.5	51.2	50.5	49.6	48.8	47.8	46.0

The sodium hydroxide was N/10, the glycin solution was 7.505 gms. glycin and 5.850 gms. sodium chloride in 1000 cc. double distilled water. The resulting 9.6 solution was identical in hydrogen ion concentration with the corresponding Lubs and Clark borate solution. The sodium hydroxide solution was practically carbonate-free and standardized against pure oxalic acid, using thymolsulphonphthalein as an indicator. The other chemicals were of the highest purity available in commercial products and were further purified in some cases.

Study of the gas exchange of the solution.—As stated previously, the method of the study of oxygen absorbed was that of McNair ('17). As the solubility of carbon dioxide must also be considered and the technique of the Van Slyke ('17) carbon dioxide apparatus is involved, time has not permitted as extensive study of the gas exchange as I had hoped. Therefore this phase of the work is reserved for a later paper.

Discussion of Data

Deamination.—All of the early work was directed toward mere color changes and the question of the specificity of the enzyme. Wolk ('12) first suggested the possibility of two distinct enzymes but offered no proof. Beijerinck ('13) offered the first definite proof that two distinct enzymes exist and suggested that nitrogen is split from the tyrosin molecule during the reaction. In the course of the isolation of certain soil organisms he noted that black spots were produced on tyrosin agar plates, wherever small transparent colonies were growing over the more deeply located colonies of *"Actinomyces."* On isolating each organism separately he was unable to find pigment production with either, but on crossing the streaks on agar the coloration was again produced. This with similar experiments led him to conclude that there were two enzymes involved, one being present in each organism.

Chodat and Schweizer ('13) showed that an enzyme extract from the potato split carbon dioxide and ammonia from glycin, leaving formaldehyde, but failed to show that this is a specific property of tyrosinase, the enzyme which gives the red and black colorations with tyrosin. Their work was wholly qualitative, carried out in solutions of varying degrees of alkalinity. Toluol was the antiseptic used, and apparently only a small amount of that. Folpmers ('16) attempted to isolate the products and succeeded in isolating benzaldehyde by the formation of benzylidene-para-nitrophenyl-hydrazone. Phenylalanin and tyrosin gave too small quantities to be determined with certainty.

I regret that I have been unable to see the work of Schweizer ('16), but, according to his reviewers, he finds the amino group split off and the side chain oxidized, as successive steps in the action of a single enzyme, tyrosinase. In the work detailed below, no deamination could be found which could be ascribed to enzyme action, although the characteristic colorations with tyrosin were obtained. A much simpler explanation for these phenomena seems to lie in considering that one is working with a mixture of enzymes, and that, in the case of the Geneva workers where the work was done largely with enzymes from a single source, a deaminase or deaminases were present as well as the enzyme, giving the red or black coloration with tyrosin solutions, which may be precipitated in the same fraction with the deaminase.

Although toluol was used to cover the surface of the solutions, in one of the flasks which stood for several days between the beginning of the experiment and the analysis, the toluol evaporated and a slight black pellicle was formed on the surface. As the solutions had been plugged with cotton, suspicion was aroused and nutrient agar plates were poured from each flask, although toluol still covered the surface of the remaining flasks. The plates were virtually pure cultures of *Bacillus sp.* which were shown to produce ammonia rapidly in Dunham's peptone solution. Hence I think the ammonia found in all of the experiments was the result of bacterial action, since it never formed a constant portion of the amino-nitrogen present nor of the nitrogen which did not enter into the reaction with nitrous acid under the conditions of a Van Slyke determination of amino-nitrogen. These bacteria have thus far withstood all attempts to kill them without injuring the enzyme, resisting thymol-chloroform, alcohol, heat, and poisons.

The percentages of nitrogen loss and gain shown in table II were computed on the basis of tyrosin nitrogen only, in order to find a common measure to study the question of deamination. This is not wholly desirable, as the solutions contained some amino- and ammonia-nitrogen extracted from the fungous flour, but as the possible bacterial action was unknown, it is felt that the figures furnish as reliable data as could be obtained under the circumstances.

The enzyme preparations were also tried with glycin, leucin, alanin, and phenylalanin. Here again the amount of ammonia found bears no relation to the amount of amino-nitrogen lost after the proper corrections have been made, and no ammonia was found in the solutions kept at ± 5° C. for two weeks, where bacterial action would be extremely slow, although a few colonies were isolated from these flasks. Here, however, the enzyme action would also be slower if it followed the Van't Hoff rule of temperature coefficients, but a characteristic tyrosinase coloration was obtained in the flasks at the end of the experiment. In all of the flasks the amount of amino-nitrogen lost and not accounted for as ammonia was small and it may probably have conjugated with

TABLE II

SHOWING LACK OF CORRELATION BETWEEN LOSS OF AMINO-NITROGEN AND GAIN OF AMMONIA-NITROGEN

Solution	Amino-nitrogen				Ammonia-nitrogen			
	Mgs. present		Loss (mg.)	†Loss (per cent)	Mgs. present		Gain (mg.)	†Gain (per cent)
	Start	End			End	Check		
Daedalea confragosa								
1.0000 gm. fungous flour 70 cc. water.......	.0835	.0617	.0217	70.4	.0051	.0026	.0025	8.2
0.5000 gm. fungous flour 75 cc. water.......	.0627	.0507	.0120	38.9	.0021	.0009	.0012	3.7
0.2500 gm. fungous flour 75 cc. water.......	.0462	.0401	.0061	19.7	.0012	.0008	.0004	1.3
0.1250 gm. fungous flour 75 cc. water.......	.0517	.0212	.0305	98.7	.0085	.0037	.0048	15.6
0.0625 gm. fungous flour 75 cc. water.......	.0499	.0451	.0048	15.5	.0092	.0030	.0063	20.4
0.1250 gm. fungous flour 75 cc. water (35°C.)	.0502	.0268	.0234	75.7	.0044	.0033	.0011	3.5
0.1250 gm. fungous flour 75 cc. water (5°C.)*	.0523	.0505	.0018	5.8	.0040	.0037	.0003	1.3
0.2500 gm. fungous flour 75 cc. water (35°C.)	.0438	.0300	.0138	44.6	.0193	.0071	.0122	39.5
0.2500 gm. fungous flour 75 cc. water (20°C.)	.0414	.0210	.0204	66.0	.0075	.0069	.0006	2.2
0.2500 gm. fungous flour 75 cc. water (5°C.).	.0459	.0434	.0025	8.1	.0044	.0044	.0000	0.0
Armillaria mellea								
0.250 gm. fungous flour 75 cc. water (35°C.)	.0346	.0284	.0062	21.0	.0052	.0031	.0021	6.7
0.250 gm. fungous flour 75 cc. water (20°C.)	.0468	.0443	.0025	8.1	.0043	.0028	.0016	5.1
0.250 gm. fungous flour 75 cc. water (5°C.).	.0459	.0434	.0025	8.1	.0033	.0033	.0000	0.0
0.250 gm. fungous flour 75 cc. water	.0424	.0296	.0128	41.4	.0044	.0018	.0026	8.3

* This was the flask in which the pellicle was noted. The two succeeding sets of flasks yielded bacteria. Probably none of the flasks of solution were wholly sterile (see discussion).

† Computed on the basis of tyrosin N present at the beginning of the experiment, 0.0309 gm.

substances present in the fungous flour. Hence I think we may conclude that there is no deamination of tyrosin by enzymes in *Armillaria mellea* and *Daedalea confragosa.*

Fate of the carboxyl and phenol groups.—Chodat and his students, as well as Folpmers, have studied the relations of the carboxyl group to a certain extent. They have concluded that the carboxyl is destroyed, carbon dioxide being evolved, and that the carbon atom next the carboxyl group forms an aldehyde group which may be further oxidized to a carboxyl, or to one alcohol and one carboxyl group by two aldehyde groups uniting with a molecule of water. On the animal side, the product of the reaction is commonly reported in the text-books as due to the formation of homogentisic acid, with the intermediate formation of a quinoid structure and the shifting of the phenol group on the ring. This gives an adequate explanation for the presence of color in the compound, but is gradually being considered untenable. Dakin ('10, '10^a, '11, '12) thinks that his theory of β-oxidation will fit the case in the normal metabolism of tyrosin, where it is completely oxidized, and that homogentisic acid formation which is said to occur in alkaptonuria is one of those peculiar anomalies of metabolism which sometimes take place. For recent work from the animal side, see also papers by Fromherz and Hermanns ('14) and Kotake ('18).

If the P_H values are plotted on a logarithmic scale and the number of cubic centimeters of standard acid or alkali necessary to bring the original solution to each successive step in hydrogen ion concentration are plotted on the millimeter scale, we obtain a graph which enables us to ascertain several important facts, bearing in mind that absolute values are obtainable only when a single pair of substances are reacting in such a way that the hydrogen ion concentration is affected. It is a well-known fact, however, that the stronger alkali or acid will inhibit the dissociation of the weaker so that it will not react readily in the presence of the stronger. Hence if the hydrogen ion concentration values do not lie too close together, it is often possible to determine the amounts of two or more acids or bases in the same solution.

Bovie ('15) presents a series of such graphs of the titration of several well-known acids and bases. At the 1916 meeting of the Botanical Society of America he suggested that a study of such titration graphs would enable one to titrate more than one substance in a solution, as the hydrogen ions of phosphoric acid may be titrated.

A study of the tables of dissociation constants by Börnstein and Roth ('12) also gives information useful in interpreting such graphs. For example, it will be noted that all of the phenol groups dissociate between P_H 9.0 and 11.0, mostly between 9.0 and 10.0. Carboxyl groups give up their ions at different points, depending upon the groups to which they are attached. Where they are attached to the same atom as an amino group, as in most of the amino acids, they dissociate between P_H 7.0 and 9.0. In acids, like aspartic and glutamic, the carboxyl at the end of the chain farthest from the amino group dissociates on the acid side of neutrality. It will be noted in the case of the few polypeptids given that polypeptid formation shifts the dissociation point toward the neutral point, i. e., toward acidity, possibly by separating the amino and carboxyl groups.

Bearing these facts in mind, let us turn our attention to tyrosin. The dissociation constants of tyrosin, as given by Kanitz ('07), show the carboxyl dissociating at P_H 8.4, the phenol group at 9.4, and the amino group at 11.8. Hence in plotting its curve, we should expect the curve to rise rapidly until 8.4 is reached, slightly less rapidly until 9.4, become horizontal until well beyond 10.0, then rise again until 11.8, finally becoming horizontal as soon as the amino group has been bound. A glance at the curve *B,* plotted from an average of all determinations, will show that this is essentially the case. Another phenomenon is also apparent. The steepness of the curve reveals to a certain extent the dissociation of the salt formed. A weak acid will form a salt which will add a larger proportion of negative ions to the positive than a strong acid which gives off hydrogen ions more rapidly, so that the resulting curve is steep. Therefore it will be seen that the acid is much weaker than the phenol group.

A study of the curve *A*, plotted from an average of many determinations, will show that one-fourth of the carboxyl has disappeared, and none of the phenol. Of all possible ways of combining tyrosin molecules, we may disregard the possibility of linkage through the two amino groups, as such compounds are notoriously unstable. Linkage of the carboxyl with the amino group, as in polypeptid formation, is unlikely, as the melanin is notoriously stable, resisting hydrolysis as very few proteins do. A distinct break will be noted in the carboxyl portion of the curve, the portion nearest neutrality dissociating at practically the same point as tyrosin and very probably representing unattacked tyrosin. It will be noted, too, that exactly the same amount of the phenol group dissociating at the same point as the phenol group of tyrosin is shown. Deducting these amounts as unused tyrosin, we see that the ratio of the carboxyl to the phenol group is 1:2. This would point to linkage not being through the phenol group, and not wholly through the carboxyls. This leaves as the only other possibility linkage of the amino group with the carboxyl, although probably not as polypeptids, for the amino groups disappear during the process without showing up as ammonia. Von Fürth and Schneider ('01) have pointed out that skatol and indol are given off when melanin is melted with pure sodium hydroxide. This would indicate the possible formation of a heterocyclic ring with the amino group, but we are hardly in a position to discuss an exact formula for it at this time.

The probability that both phenol groups belong to the same ring of the compound, which would then be considered related to compounds like homogentisic acid, reported so frequently as resulting from the tyrosin metabolism of alkaptonurics, also from seeds (Bertel, '02, Czapek, '02, Czapek and Bertel, '06), is remote. An inspection of the dissociation constants of the di-hydroxy-benzene compounds shows that the phenol groups in such cases tend to be dissociated farther toward the alkaline side of neutrality, although it is barely possible that the melanin molecule is so large that these groups are not dissociated at such low hydrogen ion concen-

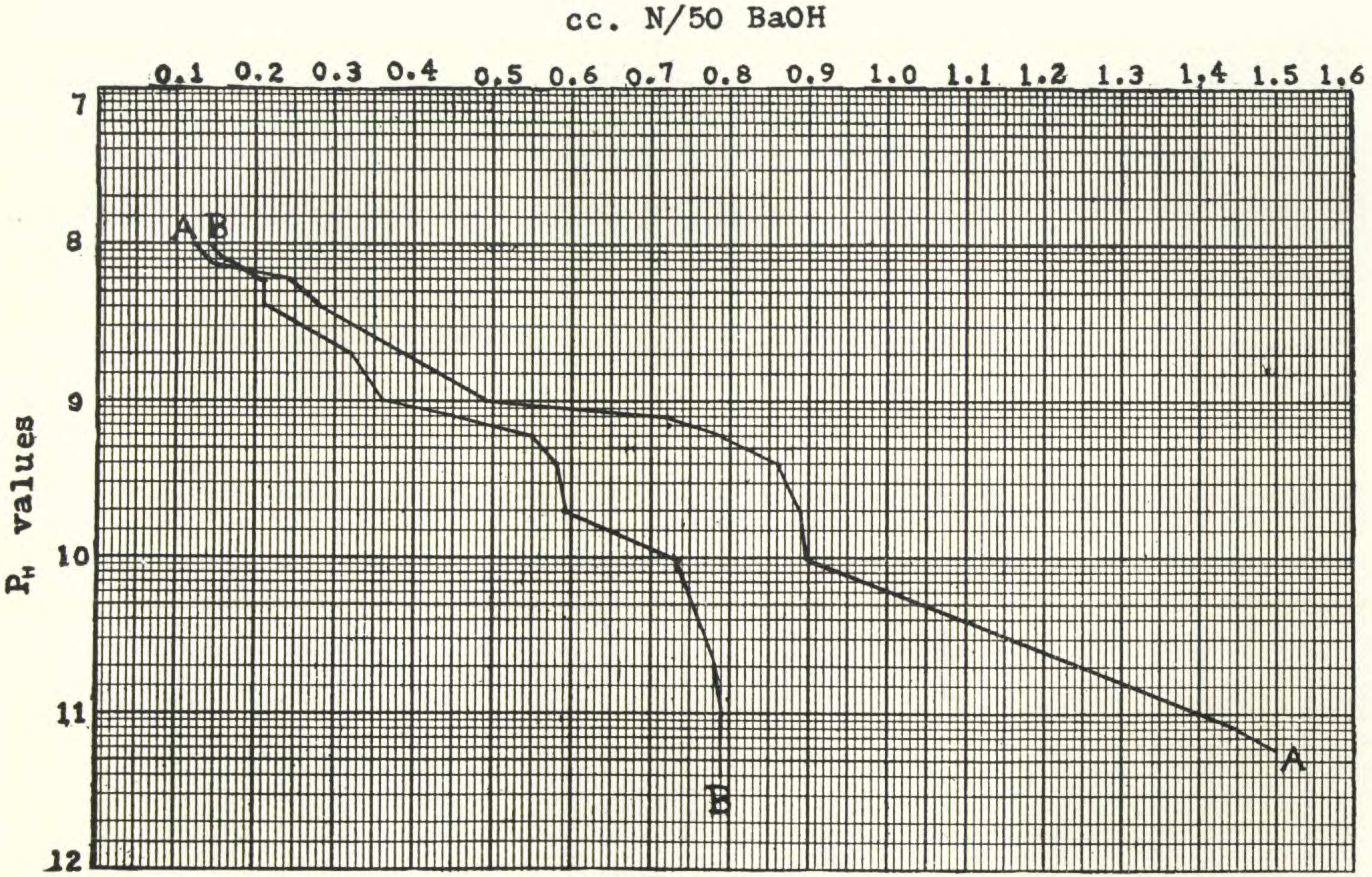

Fig. 1. Graph showing the titration of a pure tyrosin solution *(A)*, and of the solution after "melanin" formation with the acidity due to the fungous flour deducted *(B)*.

trations as usual. Data is lacking as to the possibility of these groups being partly due to compounds such as oxyphenyl lactic acid which Kotake ('18) thinks is a normal intermediary product in tyrosin metabolism, but this compound reacts so much more readily than melanin that it seems improbable that this acid is formed, since deamination is an essential feature of its formation.

TABLE III

SHOWING THE AMOUNT OF CARBOXYL AND PHENOL GROUPS

P_H values	No. cc. N/50 $Ba(OH)_2$ for 5 cc. sol. to give P_H values indicated						
	Daedalea confragosa					Armillaria mellea	Average
	.0625 gm.	0.125 gm.		0.250 gm.		0.250 gm.	
	20°C.	35°C.	5°C.	35°C.	5°C.		
8.0	0.09	0.17	0.53	0.12	......	0.00	0.18
8.2	0.11	0.22	0.58	0.15	......	0.01	0.21
8.4	0.11	0.35	0.58	0.17	......	0.03	0.25
8.6	0.15	0.36	0.58	0.36	0.24	0.15	0.30
8.8	0.15	0.36	0.72	0.36	0.26	0.26	0.35
9.0	0.18	0.46	0.77	0.38	0.28	0.30	0.39
9.2	0.24	0.66	0.84	0.38	0.40	0.50	0.50
9.4	0.25	0.79	0.98	0.55	0.38	0.52	0.58
9.6	0.34	0.79	1.04	0.60	0.34	0.53	0.61
9.8	......	0.79	1.05	0.66	0.33	....	0.71
10.0	......	0.87	1.06	0.67	0.30	0.91	0.76
10.2	......	0.91	1.07	0.67	0.30	0.94	0.78
10.4	......	0.94	1.08	0.67	0.29	0.96	0.79
10.6	......	0.96	1.09	0.66	0.29	1.00	0.80
10.8	......	0.97	1.10	0.66	0.30	1.15	0.84
11.0	......	0.97	1.10	0.66	0.29	1.18	0.84
11.2	......	0.98	1.10	0.66	0.30	1.21	0.85
11.4	......	0.98	1.11	0.66	0.29	....	....
11.6	......	0.98	1.11	0.67	0.29	....	....
11.8	......	0.99	1.12	0.67	0.29	....	....
12.0	......	1.00	1.12	0.85	0.29	....	....

All of this work should be repeated under absolutely sterile conditions, if this be possible, before one could be justified in drawing conclusions. However, the data are highly suggestive.

From my work with the cultures of bacteria isolated, I am certain that the characteristic pigment formation of tyrosin is not due to them, as they are unable to produce any color in solutions of tyrosin or tyrosin agar, either with or without the addition of carbohydrates. One organism produces ochre red or Congo pink pigment on glycerin, mannose, or sucrose broth, but not on glucose, maltose, or lactose broth.

Conclusions.—From the above data we may safely conclude: (1) that the tyrosinase reaction is not a deamination, although it is possible that deaminases may exist in the same organism with tyrosinase; (2) that the tyrosin molecule is synthesized into a larger, more complex molecule, in which part of the carboxyl groups are either split off as carbon dioxide, or more probably bound in the molecule so that it will not react with alkali.

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